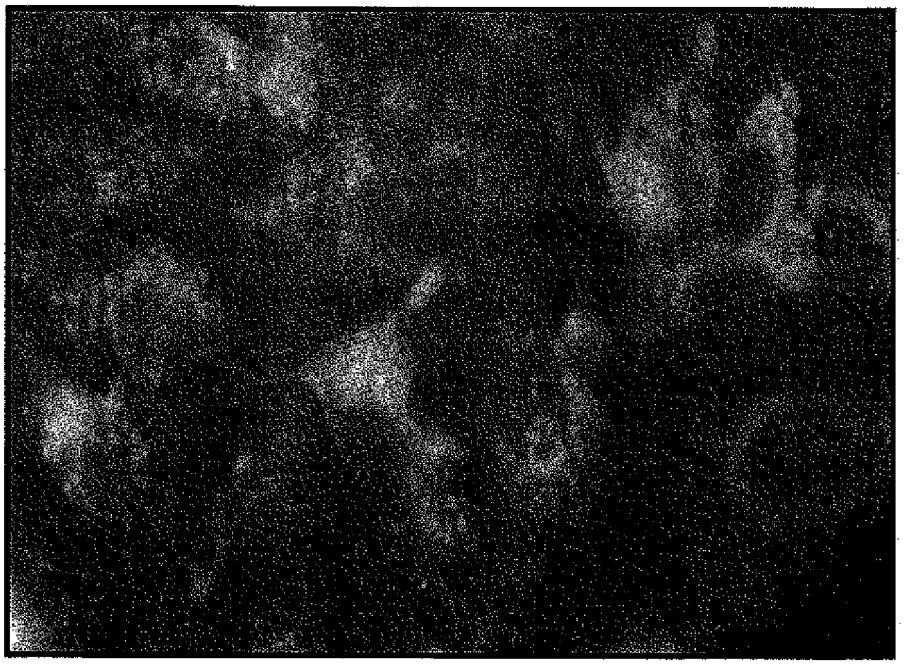
Nanotechnology for Cancer Therapy



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27 PEGylated Dendritic Nanoparticulate Carriers of Anti-Cancer Drugs

D. Bhadra, S. Bhadra, and N. K. Jain

CONTENTS

27.1 INTRODUCTION

Cancer is a leading cause of death. Irrespective of etiology, cancer is basically a disease of cells characterized by loss of normal cellular growth, maturation, and multiplication that lead to disturbance of homeostasis. The main features of cancer are (Barar 2000) excessive cell growth,

invasiveness, undifferentiated cells or tissues, the ability to metastasize or spread to newer sites and establish new growths, a type of acquired heredity where the progeny of cancer cells also retain cancerous properties, and a shift of cellular metabolism, leading to increased production of macromolecules from nucleosides and amino acids that causes an increased metabolism of carbohydrates for cellular energy, ultimately resulting in the host's illness. This illness is attributed to pressure effects as a result of local tumor growth, destruction of the organs involved by the primary growth or its metastases, and systemic effects as a result of the new growths.

A single cancerous cell surrounded by healthy tissue will replicate at a rate higher than the other cells, placing a strain on the nutrient supply and elimination of metabolic waste products. Tumor cells will displace healthy cells until the tumor reaches a diffusion-limited maximal size. Although tumor cells will typically not initiate apoptosis in a low nutrient environment, they do require the normal building blocks of cell function like oxygen, glucose, and amino acids. The vasculature that was designed to supply the now-extinct healthy tissue could not place as high a demand for nutrients because of its slower growth rate. Tumor cells will continue dividing because they do so without regard to nutrient supply, but many tumor cells will perish because the amount of nutrients is insufficient. The tumor cells at the outer edge of a mass have the best access to nutrients, and cells on the inside die creating a necrotic core within tumors that rely on diffusion to deliver nutrients and eliminate waste products. In essence, a steady state tumor size forms as the rate of proliferation is equal to the rate of cell death until a better connection with the circulatory system is created. This diffusion-limited maximal size of most tumors is around 2 mm^3 (Grossfeld, Carrol, and Lindeman 2002). To grow beyond this size, the tumor must recruit the formation of blood vessels to provide the necessary nutrients to fuel its continued expansion. It is thought that there could be numerous tumors at this diffusion-limited maximal size throughout the body. Until the tumor can gain that access to the circulation, it will remain at this size, and the process can take years.

27.2 PROBLEMS IN CANCER CHEMOTHERAPY

In the past, chemotherapy was considered as a last resort after more successful treatments like surgery and radiotherapy had failed. The main problem of cancer chemotherapy is the lack of highly selective drugs, and the rapidly dividing normal cells of the bone marrow, gut, lymphoid tissue, spermatogenic cells, fetus, and hair follicles are also killed. Most of the antineoplastic drugs act on the processes such as DNA synthesis, transcription, or the mitotic phase, and they are labeled as cell cycle phase-specific drugs (also known as phase-dependent drugs). The phase-specific drugs do not act on G_0 phase. In contrast, there are certain drugs that kill the cells during all or most phases of the cycle, and they are labeled as cell cycle phase-nonspecific drugs (also known as phase-independent drugs). The phase-specific drugs have proven to be effective in hematological malignancies and tumors with high rate of proliferation or high growth fraction (Barar 2000).

The various obstacles for drug delivery to tumors include differences in cellular morphology, tissue immunogenecity, uncontrolled rate of growth, capacity of metastasize, and poor response to chemotherapeutic agents. Furthermore, there are variations in blood flow and vessel permeability within different regions of some tissues (Reynolds 1996). Various drug delivery systems are employed for delivery of chemotherapeutic agents to neoplastic cells. This helps in targeting drugs directly into cells and preventing drug interaction with normal tissues and alleviating side effects to normal cells. Because of a lack of highly selective drugs for tumor cells and tissues and decreased penetration of drug into neoplastic cells, a number of novel drug delivery systems, prodrugs, and other chemically modified forms of drugs having altered tissue distributions were brought in use and recently reviewed for targeting to cells located in various parts of body (Brigger, Dubernet, and Couvreur 2002).

There are various means to effectively fight the tumors such as achieving targeting by avoiding reticuloendothelial system (RES); targeted delivery through enhanced permeability

and retention (EPR) effects; tumor-specific targeting; targeting through angiogenesis; targeting tumor vasculaturel; etc. (Brannon-Peppas and Blanchette 2004). Particles with longer circulation times and greater ability to target to the site of interest should be 100 nm or less in diameter and have a hydrophilic surface in order to reduce clearance by macrophages (Storm et al. 1995). Coatings of hydrophilic polymers can create a cloud of chains at the particle surface that will repel plasma proteins, and work in this area began by adsorbing surfactants to the nanoparticles surface.

Other routes include forming the particles from branched or block copolymers with hydrophilic and hydrophobic domains. One potential advantage in treatment of advance stage cancerous tissue is the inherent leaky vasculature that allows for greater accumulation of colloidal systems. The check the defective vascular architecture, created as a result of the rapid vascularization necessary to serve fast-growing cancers, coupled with poor lymphatic drainage allows an enhanced permeation and retention effect (EPR effect) (Sledge and Miller 2003). The ability to target treatment to very specific cancer cells also uses a cancer's own structure in that many cancers over express particular antigens, even on their surface. This makes them ideal targets for drug delivery as long as the targets for a particular cancer cell type can be identified with confidence and are not expressed in significant quantities anywhere else in the body. Tumor-activated prodrug therapy uses the approach that a drug conjugated to a tumor-specific molecule will remain inactive until it reaches the tumor (Chari 1998). These systems would ideally be dependent on interactions with cells found specifically on the surface of cancerous cells and not the surface of healthy cells. Most linkers are usually peptidase cleavable or acid labile but may not be stable enough in vivo to give desirable clinical outcomes.

Limitations also exist because of the lower potency of some drugs after being linked to targeting moieties when the targeting portion is not cleaved correctly or at all. For example, adriamycin-conjugated poly(ethylene glycol) linker with enzymatically cleavable peptide sequences (alanyl-valine, alanyl-proline, and glycyl-proline) or using monoclonal antibodies has shown a greater selectivity to cleavage at tumor cells (Suzawa et al. 2002). A number of targeted cancer treatments using antibodies for specific cancer types have been approved by the U.S. Food and Drug Administration like rituximab, trastuzumab, gemtuzumabozogamicin, alemtuzumab, ibritumomab tiuxetan, gefitinib, etc. (Abou-Jawde et al. 2003).

27.3 NANOPARTICLES IN CANCER CHEMOTHERAPY

Nanotechnology applied to cancer treatment may offer several promising advantages over conventional drugs. Nanoscale devices are two orders of magnitude smaller than tumor cells, making it possible for them to directly interact with intracellular organelles and proteins. Because of their molecule-like size, nanoscale tools may be capable of early disease detection using minimal amounts of tissue, even down to a single malignant cell (NCI 2005). These tools may not only prevent disease by monitoring genetic damage, but also treat cells in vivo while minimizing interference with healthy tissue. By combining different kinds of nanoscale tools on a single device, it may be possible to run multiple diagnostic tests simultaneously (www.math.uci.edu/~cristini/publications/nanochap.pdf). In particular, it is hoped that cancer drug therapy involving nanotechnology will be more effective in targeting malignant cells and sparing healthy tissue. In this regard, the role of nanoparticles loaded with chemotherapeutic drugs has been receiving much attention. Research and development in this area is expected to dramatically increase in importance in the coming years. In general, nanoscale drug delivery systems (Figure 27.1) for chemotherapy can be divided into two categories: polymer- and lipid-based (Langer 2000; Sahoo and Labhasetwar, 2003).

Polymers are usually larger than lipid molecules, and they form a solid phase such as polymeric nanoparticles, films, pellets, dendrimers; whereas, lipids form a liquid (or liquid crystalline phase) such as liposomes, cubersomes, micelles, and other emulsions (Feng and Chien 2003). Whereas

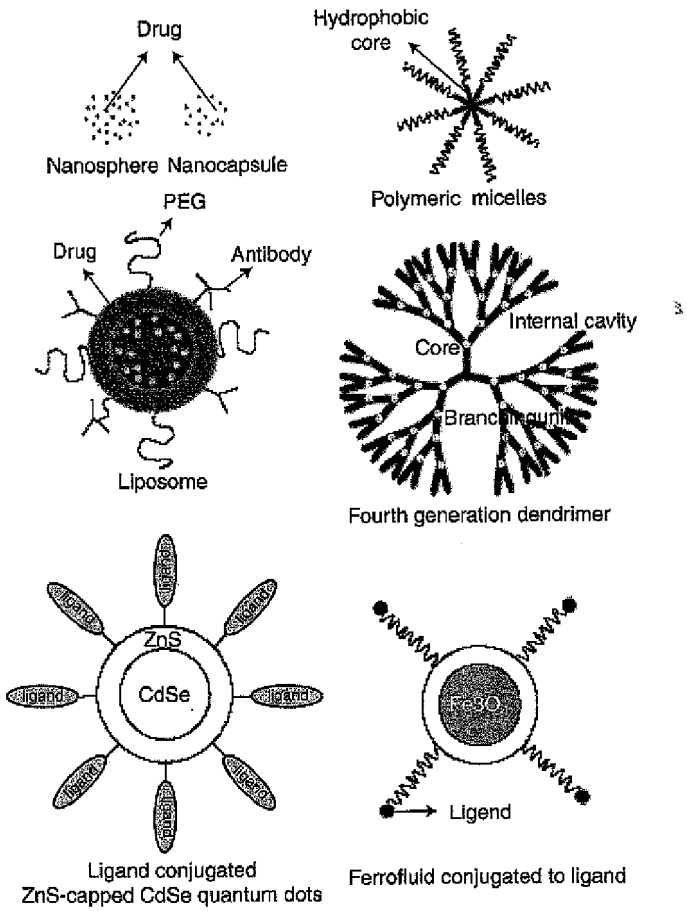

FIGURE 27.1 Representation of different nanoparticulate carriers used for drug delivery. (Adapted from Sahoo, S. K. and Labhasetwar, V., *Drug Discov. Today*, 8, 2003, www.drugdiscoverytoday.com. With permission.)

polymer-based systems are considered biologically more stable than lipid-based systems, the latter are generally more biocompatible. Polymer-based systems might possess good drug targeting ability because their uptake may be different for cells in different tissues (Maruyama 2000). In fact, Feng and Chien (2003) have suggested that a combination of polymer- and lipid-based systems could integrate their advantages while avoiding their respective disadvantages. An example of such a nanoparticle would be a liposomes-in-microspheres (LIM) system where drugs are first loaded into liposomes and then encapsulated into polymeric microspheres. This way, both hydrophobic and hydrophilic drugs can be delivered in one nanoparticle. The bioactivity of peptides and proteins would be preserved in the liposomes whose stability is protected by the polymeric matrix (Feng and Chien 2003).

Chemotherapy using nanoparticles has been studied in clinical trials for several years, and numerous studies have been published in this regard (Kreuter 1994). Two liposomally delivered drugs are currently on the market: daunorubicin and doxorubicin (Massing and Fuxius 2000). These encapsulated drugs can be formulated to maximize their half-life in the circulation. For example, a stealth version of liposomal doxorubicin coated with polyethylene glycol to reduce its uptake by the RES can extend its half-life in blood for up to 50–60 h (CCO Formulary 2003).

27.3.1 Challenges of Nanotechnology

The difficulties faced by nanotechnology in the service of clinical medicine are numerous. These difficulties should be kept in mind while considering chemotherapeutic treatment involving nanotechnology and the potential role of biocomputation. First, there are basic physical issues that matter on a small scale. Because matter behaves differently on the nanolevel than it does at micro- and macro levels, most of the science at the nanoscale has been devoted to basic research, designed to expand understanding of how matter behaves on this length scale (www.math.uci.edu/~cristini/publications/nanochap.pdf). Because nanomaterials have large surface areas relative to their volumes, phenomena such as friction are more critical than they are in larger systems. The small size of nanoparticles may result in significant delay or speed-up in their intended actions. They may accumulate at unintended sites in the body. They may provoke unexpected immune system reactions. Cells may adapt to the nanoparticles, modifying the body's behavior in unforeseen ways (www.math.uci.edu/~cristini/publications/nanochap.pdf).

The efficacy of nanoparticles may be adversely affected by their interaction with the cellular environment. For instance, the RES may clear nanoscale devices, even stealth versions, too rapidly for them to be effective because of the tendency of the RES to phagocytose nanoparticles (Kreuter 1994). Nanoparticles can be taken up by dendritic cells (Elamanchili et al. 2004) and by macrophages (Cui, Hsu, and Mumper 2003). RES accumulation of nanoparticles could potentially lead to a compromise of the immune system. On the other hand, larger nanoparticles may accumulate in larger organs, leading to toxicity. Perhaps the biggest issue of all is that the physically compromised tumor vasculature may prevent most of the nanodevices from reaching the target cells by vascular transport or diffusion. Alterations in the tumor vasculature may adversely affect the convection of the nanodevices in the blood stream (Brigger, Dubernet, and Couvreur 2002). Local cell density and other stromal features may hamper drug or nanodevice diffusion through tumoral tissue.

27.4 POLY(ETHYLENE GLYCOL) CONJUGATION

Poly(ethylene glycol) is a non-toxic water-soluble polymer that resists recognition by the immune system. The term PEG is used for poly(ethylene glycol) that is often referred for polymer chains with molecular weight (MWs) below 20,000 Da, and poly(ethylene oxide) (PEO) refers to higher MW polymers (Harris et al. 1984; Peppas et al. 1999). These are homogeneous polymers of $M_w/M_n < 1.1$ represented by the formula $HO(CH_2CH_2O)_nH$ and is called as poly(oxy-1, 2-ethanediyl), or α-hydro-ω-hydroxy-poly(ethylene glycol). It is an additional polymer of ethylene oxide and water, where n represents the average number of oxy-ethylene groups. These are generally soluble in water, alcohol, acetone, and chloroform, but miscible with glycols and practically insoluble in ether. These are also known as Macrogols (Reynolds 1996) that are generally of higher molecular weights such as Macrogols of 20,000 Da. The pH of 5% solution is about 4–7.5.

The aqueous solutions of PEGs can be sterilized by filtration, autoclaving, etc. and are needed to be stored in airtight containers. The PEGs are relatively less toxic, but toxicity, if any appears to be greatest with macrogols of lower molecular weights. On topical administration, it may cause stinging, especially to mucous membrane. Such administration of PEGs may be associated with hypersensitivity reactions such as urticaria, and local gastrointestinal discomfort, bloating and nausea, abdominal cramps, vomiting and irritation, may result on its use in bowel cleansing preparation. Macrogols demonstrate oxidizing ability, leading to incompatibility with activity of bacitracin or benzyl-penicillin that may get reduced in macrogol bases. Estimated acceptable daily intake may be upto 10-mg/kg body weight (Reynolds 1996). Its presence in aqueous solution has no deleterious effect on protein conformation or activity of enzymes. It exhibits rapid clearance from the body, and it has been approved for a wide range of biomedical applications. PEG may transfer its properties to other molecules when it is covalently bound to that molecule. This can result in modification of number of properties of molecules as illustrated later on (Zalipsky 1995).

PEG has polyether backbone that is inert in biological environment as well as in most chemical reaction conditions as in chemical modifications and/or conjugation reactions. The chemical derivatisation of end groups of PEG is an essential first step in preparation of bioconjugates. The various properties are altered because of polyethylene glycol conjugation that is discussed in the following sections. PEG conjugation was tried and found most applicable in case of many nanoparticulate systems, using activated PEGs with appropriate spacers.

27.4.1 Derivatization and Activation of Poly(Ethylene Glycol)

Poly(ethylene glycol) has two equivalent hydroxyl groups, so this could act as potential cross-linking agent for any systems to which it is attached. These hydroxyl groups can be attached to various bio-active species by covalent coupling using number of simple PEG analogues. Some important stable analogues are bromo, amino, aminoethyl, carboxymethyl, succinimidosuccinate, tosylate, mesylate, aldehyde, octadecylamine, monopalmitate, stearoyloxy derivative of PEG, or monomethoxy-PEG (MPEG) (Buckmann and Morr 1981; Harris et al. 1984). These were prepared for reactions with sensitive bio-active systems under mild conditions.

One very important derivative that has been used in number of derivatisation reactions that has one hydroxyl group blocked is monomethyl-ether of polyethylene-glycol or monomethoxy-polyethylene-glycol (MPEG), i.e., considered equivalent in terms of reaction for formation of PEG derivatives. This was generally brought in use for conjugation to bioactive species. It is generally used when multiple chains of polymers had to be linked to the intended substrates. Because of structural simplicity and possession of only one-derivatizable end group, the use of MPEG minimizes cross-linking possibilities and leads to improved homogeneity of conjugate. Therefore, it usually is a starting material of choice for the covalent modification of proteins, biomaterials, particulates, lipids, drugs, etc. (Zalipsky 1995).

$$\mathrm{MeOH} + \mathrm{H(OCH_2CH_2)_nOH} \rightarrow \mathrm{MeO(CH_2CH_2O)_nH}$$

Commercially available MPEGs are often contaminated with significant amounts (equivalent to 25%) of dihydroxy-terminated polymers and may be purified by chromatography and ethyl ether precipitation. The use of these polymers as starting materials of choice for covalent modification of proteins, biomaterials, and particles is dependent for not forming cross-linked conjugates (Harris et al. 1984).

In most cases, commercially available MPEGs of 2000–5000 MWs are used for preparation of number of reagents. MPEG-linked and based electrophiles were synthesized and used for linking with number of available attachment sites, e.g., amino acids and other nucleophilic groups. These are called activated PEGs (Zalipsky 1995). The intermediate linkers are called spacers, linking to bioactive groups, especially proteins and enzymes. These generally require mild conditions of reactions and specific properties of reactants for binding.

In order of reaction and based on ease of introduction, these can roughly be arranged as follows: arylating agents > acylating agents > alkylating agents (Zalipsky 1995). For example, tresylates, succinimidyl succinate derivatives, succinimidyl ester of carboxymethyl PEG, oxy-carboxylamino acid derivative of PEG, acetaldehyde, and hydrazide derivatives were mainly used for PEGylation of proteins and enzymes.

27.4.2 Properties of Poly(Ethylene Glycol) Conjugates

This group reviewed various properties affected by PEGylation of carriers (Bhadra et al. 2002). PEGylation has pronounced effects on various parameters of drug delivery systems (Katre 1993; Zalipsky 1995). As far as bio-distribution and pharmacokinetic, PEG conjugation increases blood circulation half-life, by reducing the tissue distribution, RES, liver, spleen, and macrophage uptake

of the carriers. (Veronese et al. 1989; Papahadjopoulus et al. 1991; Phillips et al. 1999). PEG coatings alter solubility of the systems (Choi et al. 1998;Kim et al. 1999). It reduces the toxicity by decreasing the release and contact of active constituent, decreasing immunogenicity, cell-adherence, thrombogenicity, and protein adsorption. PEGylation can also lead to stabilization of delivery systems. It can also improve utilization as drug a delivery system by increasing drug loading per system, improving drug targeting as in case of liposomes by diffusion controlling mechanisms, and promoting its use as a micellar delivery system. This process can sustain and control drug delivery safely and appropriately by decreasing burst effects and by decreasing drug leakage and increasing stability of system. It can improve transfection capability (Choi et al. 1998). This can increase tumor uptake (Papahadjopoulus et al. 1991; Ishida et al. 1999) of drug and delivery systems.

Manipulation of the upper layers (corona) of nanoparticles or dendrimers confers advantageous properties to such particles, e.g., increased solubility and biocompatibility (McNeil 2005). Attaching hydrophilic polymers to the surface such as PEG greatly increases the hydration (i.e., solubility) of the nanoparticles and can protect attached proteins from enzymatic degradation when used for in vivo applications (Bhadra et al. 2002; Harris and Chess 2003). Nanoparticles with hydrophilic polymers such as PEG attached to their surface can act as a platform for lipophilic molecules, and they can overcome the solubility barrier. Insoluble compounds can be attached, adsorbed, or otherwise encapsulated in the hydrated nanoparticles. The surface addition of PEG and other hydrophilic polymers also increases the in vivo compatibility of nanoparticles. When intravascularly injected, uncoated nanoparticles are rapidly cleared from the bloodstream by the reticuloendothelial system (RES) (Brigger, Dubernet, and Couvreur 2002). Nanoparticles coated with hydrophilic polymers have prolonged half-lives, believed to result from decreased opsonization and subsequent clearance by macrophages (Moghimi and Szebeni 2003).

Cancer therapy has benefited from the use of liposomal doxorubicin, a formulation that again increases the therapeutic index of the active agent through a combination of passive tumor targeting and reduced toxicity (Gabizon and Martin 1997). In this case, coating the liposome with PEG significantly decreases uptake by macrophages and allows the liposomes to concentrate in tumors by escaping from the leaky vasculature surrounding solid tumors (Dvorak et al. 1988) through EPR effect (Maeda 2001). The surface chemistry of the nanoshells can be modified with PEG to increase biocompatibility and with sulfide-based linkers to allow the particles to be functionalized with targeting ligands.

27.5 DENDRIMER AS DRUG DELIVERY SYSTEM: SELECTION, APPLICABILITY, AND RATIONALITY

Dendrimers are a relatively new class of polymers with structures that depart rather dramatically from traditional linear polymers that are built from so-called AB monomers (Newkome, Moorefield, and Vogtle 1996). Because they are built from AB_n, monomers (where n is usually 2 or 3), dendrimers are highly branched and have three distinct structural features: a core, multiple peripheral (end-) groups, and branching units that link the two. The peripheral groups and branching units are together called dendrons, or informally, wedges. Branching units used to date have contained virtually every type of functional group, ranging from those comprising pure hydrocarbons or aromatic groups to modified carbohydrates and nucleic acids. Dendrimers are iteratively synthesized so that the number of intervening branching units between the core and one end-group (i.e., the number of layers), called the generation number, is determined by the number of synthetic cycles. In the commercially available poly(amidoamine) (PAMAM) dendrimers, ammonia represents the zeroth generation (Tomalia, Naylor, and Goddard 1990). As the generation number increases from one to seven, the number of peripheral groups follows the geometric series 3, 6,

12,...,192. These peripheral groups largely control the solubility of the compound so that even with highly hydrophobic internal units, a dendrimer will dissolve in water if the end groups are sufficiently hydrophilic. As will be discussed here, this feature and the high local concentration of end groups enable a number of applications in bio-organic chemistry. Dendrimers represent a new class of highly branched polymers whose interior cavities and multiple peripheral groups facilitate potential applications in biomedicine and bio-organic chemistry (Kim and Zimmerman 1998). Major advances in the past years were made in the synthesis and study of new carbohydrate, nucleic acid, and peptide dendrimers as well as in the use of dendrimers as magnetic resonance imaging contrast agents, agents for cellular delivery of nucleic acids, and scaffolds for biomimetic systems.

Researchers are now trying to synthesize dendrimer clusters for targeted therapy. Scientists at the University of Michigan have devised a method to easily create multipurpose molecules for use in targeted anti-cancer therapy (NCI 2005). By attaching single-stranded DNA linkers to dendrimers, the researchers could bridge together individual dendrimer subunits with specific functions and create a multifunctional dendrimer cluster. This bridging technique could potentially lead the way to developing customized therapies for individual patients. Dendrimers are promising candidates for use as the base of anti-cancer drugs to which functional molecules can be attached.

Ideally, an anti-cancer therapeutic agent would have multiple functional groups such as a targeting molecule to bind a cell, a radioactive compound to kill the cell, and a fluorescent probe so the process can be imaged. However, there are problems synthesizing these compounds, and different drugs would have to be designed for each different tumor with this approach. Dr. James Baker, Jr. and colleagues improved compound synthesis through a building block approach in one NCI-funded study. They created two single-function dendrimers; one with a molecule to bind folate receptors and the other with a fluorescein molecule for imaging. They then added complimentary 34-base stretches of single-stranded DNA to each, and the two dendrimers conjugated through base pairing. Using fluorescence imaging, they observed that the dendrimer cluster specifically bound to a cancer cell line over expressing the folate receptor. Compared with traditional chemistry, DNA-linked dendrimers could provide a more effective way to mix and match different therapeutic combinations to better treat individual patients.

Dendrimers represent one nanostructured material that may soon find its way into medical therapeutics. Starburst dendrimers are tree-shaped synthetic molecules with a regular branching structure emanating outward from a core that forms nanometer by nanometer with the number of synthetic steps or generations dictating the exact size of the particles, typically a few nanometers in spheroidal diameter (Freitas 2005). The peripheral layer can be made to form a dense field of molecular groups that serve as hooks for attaching other useful molecules such as DNA that can enter cells while avoiding triggering an immune response unlike viral vectors commonly employed today for transfection. Upon encountering a living cell, dendrimers of a certain size trigger a process called endocytosis where the cell's outermost membrane deforms into a tiny bubble or vesicle. The vesicle encloses the dendrimer that is then admitted into the cell's interior. Once inside, the DNA is released and migrates to the nucleus where it becomes part of the cell's genome. The technique has been tested on a variety of mammalian cell types (Kukowska-Latallo et al. 2000) and in animal models (Vincent et al. 2003) though clinical human trials of dendrimer gene therapy remain to be done.

Glycodendrimer nanodecoys have also been used to trap and deactivate some strains of influenza virus particles (Reuter et al. 1999; Landers et al. 2002). James Baker's group at the University of Michigan is extending this work to the synthesis of multi-component nanodevices called tecto-dendrimers built up from a number of single-molecule dendrimer components (http://nano.med.umich.edu/projects/Dendrimers.html; Quintana et al. 2002). Tecto-dendrimers have a single core dendrimer surrounded by additional dendrimer modules of different types, each type designed to perform a function necessary to a smart therapeutic nanodevice (Figure 27.2). Baker's group has

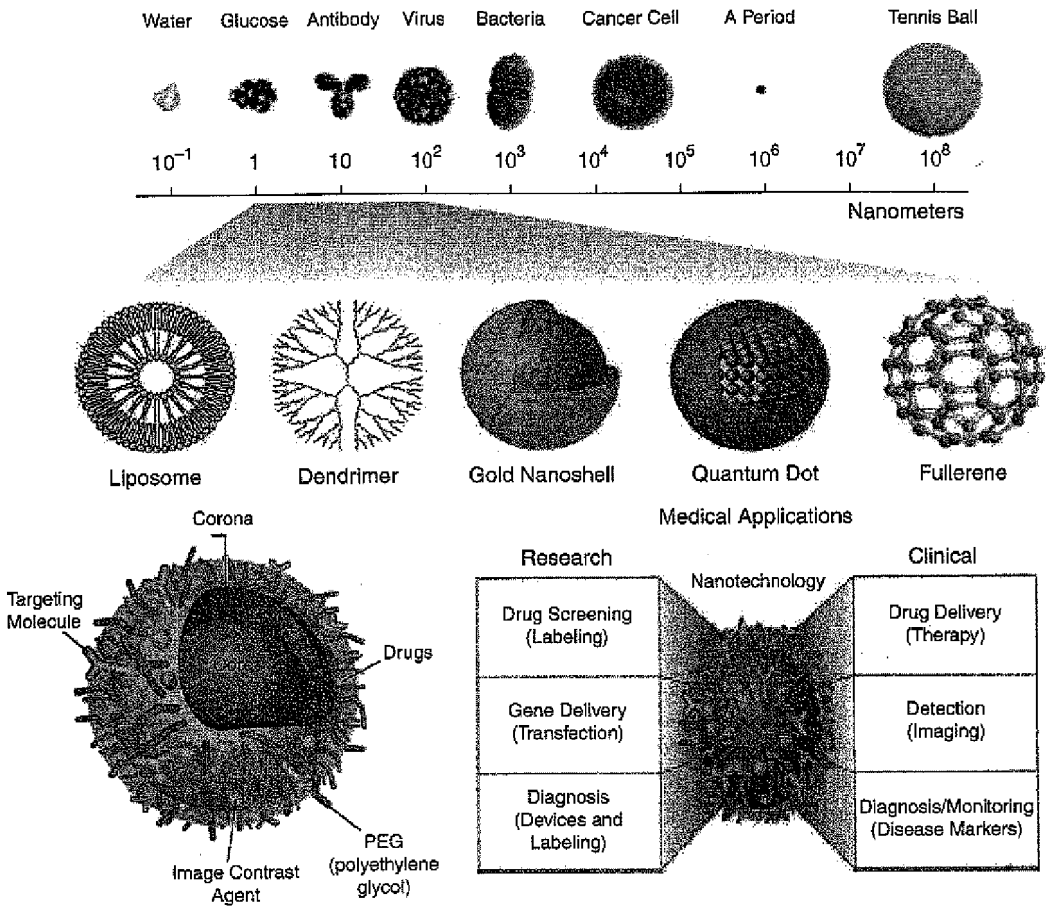

FIGURE 27.2 Morphological and size-based comparison of various novel drug delivery carriers such as liposome, dendrimer, gold nanoshell, quantum dot, fullerene, and nanometric scale of representation of various similar systems. (Adapted from McNeil, S. E., *J. Leukoc. Biol.*, 78, 1–10, 2005. With permission.)

built a library of dendrimeric components from which a combinatorially large number of nanodevices can be synthesized.

The initial library contains components that will perform the following tasks: diseased cell recognition, diagnosis of disease state, drug delivery, report location, and report outcome of therapy. By using this modular architecture, an array of smart therapeutic nanodevices can be created with little effort. For instance, once apoptosis-reporting, contrast-enhancing, and chemotherapeutic-releasing dendrimer modules are made and attached to the core dendrimer, it should be possible to make large quantities of this tecto-dendrimer as a starting material. This framework structure can be customized to fight a particular cancer simply by substituting any one of many possible distinct cancer recognition or targeting dendrimers, creating a nanodevice customized to destroy a specific cancer type and no other while also sparing the healthy normal cells. These nanodevices were synthesized using an ethylenediamine core with folic acid, fluorescein, and methotrexate covalently attached to the surface to provide targeting, imaging, and intracellular drug delivery capabilities (Freitas 1999). The "targeted delivery improved the cytotoxic response of the cells to methotrexate 100-fold over free drug" (Quintana et al. 2002).

At least a half dozen cancer cell types have already been associated with at least one unique protein that targeting dendrimers could use to identify the cell as cancerous, and as the

genomic revolution progresses, it is likely that proteins unique to each kind of cancer will be identified, allowing Baker to design a recognition dendrimer for each type of cancer (http://nano.med.umich.edu/projects/Dendrimers.html). The same cell-surface protein recognition-targeting strategy could be applied against virus-infected cells and parasites. Molecular modeling has been used to determine optimal dendrimer surface modifications for the function of tecto-dendrimer nanodevices and to suggest surface modifications that improve targeting (Quintana et al. 2002).

NASA and the National Cancer Institute have funded Baker's lab to produce dendrimer-based nanodevices that can detect and report cellular damage because of radiation exposure in astronauts on long-term space missions (Sparks 2002). By mid-2002, the lab had built a dendrimeric nanodevice to detect and report the intracellular presence of caspase-3, one of the first enzymes released during cellular suicide or apoptosis (programmed cell death) that is one sign of a radiation-damaged cell. The device includes one component that identifies the dendrimer as a blood sugar so that the nanodevice is readily absorbed into a white blood cell and a second component using fluorescence resonance energy transfer (FRET) that employs two closely bonded molecules. Before apoptosis, the FRET system stays bound together, and the white cell interior remains dark upon illumination. Once apoptosis begins and caspase-3 is released, the bond is quickly broken, and the white blood cell is awash in fluorescent light. If a retinal scanning device measuring the level of fluorescence inside an astronaut's body reads above a certain baseline, counteracting drugs can be taken.

27.5.1 Differences Between Hyperbranched Structures and Dendrimers

Dagani (1996) discussed and compared hyperbranched polymers and dendrimers. Hyperbranched polymers have highly branched structure, but unlike dendrimers, their structure is neither regular nor highly symmetrical. The dendrimers were obtained by careful stepwise growth of successive layers or generations. Hyperbranched polymers are obtained in a single step by polycondensation of A_2B monomers that contain two reactive groups of type A and one of type B. Functional groups A and B are selected in a way that they react with each other to form a covalent bond. Structure of hyperbranched polymers is thought to be intermediate between those of linear polymers and dendrimers. Because of steric constraints and statistical nature of coupling steps, hyperbranched polymers have irregular structures as some of the A groups remain unreacted, leading to incorporation of linear segments (i.e., monomers units coupled at two rather than three points) (Frechet 1994). Voit, Turner, and Mourey (1993) showed that viscosity-MW behavior of the hyperbranched polymers does not show a maximum as observed in the case of dendrimers. Instead, they obey the Mark–Hownick–Sakunada relation. Although their viscosities are anomalously lower when compared to linear polymers of comparable size. Analogous to dendrimers, A_2B hyperbranched polymers had essentially same number of reactive groups as they have monomeric units. However, unlike dendrimers, these functional groups are not all located at chain ends (Frechet 1994).

27.5.2 Classification of Dendrimers

The dendrimers that are most studied and reported up to higher generations (Bosman, Janssen, and Meijer 1999) can be classified chemically viz. peptide dendrimers that were patented and described upto higher generations by Denkewalter in the early 1980s. Those can well be characterized by size-exclusion chromatography only. PAMAM dendrimers are another class of dendritic structures that have been thoroughly investigated and had received wide spread attention. These were divergently constructed, e.g., PAMAM described by Tomalia and arborol systems of Newkome. Polypropyleneimine dendrimers were produced by Vogtle by divergent synthesis. It was also produced by heterogeneously catalyzed hydrogenation by De Brabander-van-den Berg and Meijer (1993) and is also studied widely. Frechet produced Poly-ether dendrimers by convergent procedures (Wooley et al. 1994). These also include Poly(aryl ether) dendrimers. Moore produced Phenyl acetylene dendrimers by convergent approach. Tetrathiofulvalene $(TTF)_{21}$-glycol

dendrimers are another class of dendrimers that are intra-dendrimer aggregates of TTF cation radicals (Christensen et al. 1998). These are evident by spectrochemical studies of partially oxidized TTF units. These were prepared by convergent approaches. Pentaporphyrin is another starburst porphyrin polymer that is considered as a first generation dendrimers (Norsten and Branda 1998). These had hybrid properties of porphyrin and dendrimers where ether linkages are found for iterations. Aryl ester monodispersed dendrimers based on 1, 3, 5-benzene tricarboxylic acid were initially described by Miller, Kwock, and Neenan (1992) and prepared by convergent synthesis. This was possibly used for MWs standards as polymeric rheological modifier, molecular inclusion hosts, etc. Two types of dendrimeric nucleic acids have been studied, viz. those that are covalently connected, and those that self-assemble via complementary base-pairing schemes (Kim and Zimmerman 1998). An example of a covalent DNA dendrimer was reported, wherein a second-generation phosphodiester-based dendrimer derived from pentaerythritol held nine pentathymidylate units on the end-groups and either a 15-mer or 26-mer at the core.

Additionally, many other types of interesting, valuable, aesthetically pleasing dendritic systems have been developed such as Dendrophanes that were first described by Diederich and coworkers. They called these phenyl methane-based cyclophane. These were designed as globular proteins. Metallodendrimers include Ruthenium-terpyridine complexed dendrimers by Newkome and coworkers, dendritic iron (II) complexes by Chow and coworkers, zinc-porphyrin dendrimers by Diederich and coworkers, etc. and they contain metal complexed in dendrimer structure. Polyamino phosphine containing dendrimers are phosphorous containing dendrimers. Mesogen functionalized carbosilane dendrimer involves functionalization by 36 mesogenic units attached through C-5 spacer to liquid crystalline dendrimer that form smectic-A phase in a temperature range of 117–130°C. Dendritic box was based on construction of a chiral shell of protected amino acids onto polypropyleneimine dendrimers with 64 amino end groups. These monodispersed dendritic containers of nanometric dimensions have physically entrapped or locked in guest molecules (Jansen et al. 1994; Jansen and Meijer 1995). Toyokuni et al. (1994) described an example of carbohydrate dendrimers for tumor-associated carbohydrate antigens. They linked it without the use of macromolecular carrier or an adjuvant, and they conjugated it with starburst PAMAM dendrimers to elicit antibody responses.

Dendrimeric systems can also be classified on the basis of physical characteristics of the system such as simple dendrimers having simple monomeric units; liquid crystalline dendrimers having mesogenic liquid crystalline substances; chiral dendrimers having optical activities; and micellar dendrimers having unimolecular micellar structures or water-soluble hyperbranched dendrimers of polyphenylene, etc. Hybrid dendrimers are combinations of dendritic and linear polymers in hybrid block or graft copolymers forms, whereas amphiphilic dendrimers are a class of globular dendrimers having unsymmetrical, but highly controlled, distributions of chain end chemistry. These are oriented at interfaces forming interfacial liquid membranes for stabilizing aqueous organic emulsion. These may be oriented under influence of external stimulus, e.g., electric field,

27.5.3 Features of Dendrimers as Nanodrugs

The various features of dendrimeric formulations in use include cost and ease of manufacture for cost effective (compared with small molecule drugs), and such formulations can be scaled-up for Current Good Manufacturing Practices (cGMP) guidelines manufacture. These are effective and active against a wide range of diseases and for their novel modes of action. Also at therapeutic doses, the dendrimers are safe and stable as solids, and they are thus available in a variety of Pharmaceutical formulations. The formulations are reproducible and defined as far as identity is concerned. They appear in most cases as white to off-white powder. Starpharma-like Pharmaceuticals (www.starpharma.com/framemaster.htm) are creating value from such dendrimer-based nanotechnology. Their core business strategies include developing high-value dendrimer nanodrugs to address unmet market needs, partnering with pharmaceutical companies to create new

opportunities and solutions to problems with the application of dendrimer nanotechnology, extending core skills and know-how through licensing and partnering with others, and investing in non-pharmaceutical applications of dendrimer technology. Rational drug design is being used by the biotechnology and pharmaceutical industry to design novel drugs based on known biological targets (e.g., receptors involved in the pathogenesis of disease). In this context, Starpharma's dendrimer technologies offer a unique ability to design functionalized, polyvalent dendrimers as defined species for specific biological targets.

The dendrimers are being eyed as drug-delivery agents, micelle mimics, and nanoscale building blocks. There are also added possibilities for grafting of active groups on surface of dendrimer by using cross-linkers. Also fatty acids, and polymers such as poly(ethyleneglycol) (PEG), etc. can be attached to dendrimers due to –COOH and $-NH_2$ terminal active groups present on its surface. PEG coatings, also called as PEGylation, could also have many possible numerous advantages so could be selected for coating (Zeng and Zimmerman 1997). Gitsov and Fréchet (1996) published some preliminary results on macromolecules that change shape when the polarity of the solvent changes. These macromolecules consist of four long PEG chains extending out from a central carbon atom. Each of these hydrophilic PEG arms is terminated with a hydrophobic wedge-like dendritic group based on 3,5-dihydroxybenzyl alcohol. The arms are long enough and flexible enough to allow this hybrid star to assume several very different conformations in solution. In tetrahydrofuran, the hybrid star forms a unimolecular micelle that has a hydrophilic core of tightly packed PEG arms surrounded by a loose hydrophobic shell of dendritic wedges. In chlorinated solvents such as chloroform where both building blocks are readily soluble, the PEG arms (and their dendritic extremities) extend outward, making the core more accessible. In polar or aqueous media such as methanol, the hydrophilic PEG arms loop around the hydrophobic dendritic wedges, pushing the wedges into the star's core and leaving an outer PEG layer exposed to the outside world. The hybrid stars rearrange their two types of components in this way to minimize the overall free energy of the system.

A novel hybrid star prepared in Fréchet's lab at the University of California Berkeley changes its conformation when the polarity of the solvent medium changes. In THF (tetrahydrofuran), the hydrophilic PEG core is compact, whereas in chloroform, it is more extended and open. In methanol, the hydrophilic PEG arms envelope the hydrophobic dendritic wedges, leading to two possible conformations. In one, the dendritic wedges are tightly packed toward the middle of the micelle with the PEG arms forming loops in the surrounding medium. In the other arrangement, each dendritic moiety is individually wrapped in a PEG arm and somewhat extended into the medium. Freemantle (1999) also explored blossoming of dendrimers in a conference that explored design, synthesis, structure, and potential applications of highly branched macromolecules.

27.6 APPLICATIONS OF PEGYLATED DENDRIMERS

Dendrimers are synthetic, nanoscale structures (1–100 nm) types of polyvalent pharmaceuticals (www.starpharma.com/framemaster.htm) that can be tailored for many pharmaceutical applications. Specialized chemistry techniques allow for precise control of the physical and chemical properties of dendrimers. They are constructed in a series of controlled steps (see below) that increase the number of small branching molecules around a central core molecule. The final generation of molecules added to the growing structure makes up the polyvalent surface of the dendrimer. The core branching and surface molecules are chosen to give desired properties and functions. Dendrimers allow researchers, for the first time, to produce highly defined and biocompatible nanoscale objects built from the bottom up. This high definition enables unique functionality in life sciences applications. For readers interested in a historical account, terminology, nomenclature, or other background material, an excellent monograph by Newkome, Moorefield, and Vogtle (1996) is recommended. Zeng and Zimmerman (1997) broadly reviewed

applications of dendrimers in bioorganic chemistry that have been reported in the past year, and, although somewhat different in focus, it updates earlier reviews published. An excellent review of dendrimers that act as biological mimics was also published by Smith and Diederich (1998). Beyond discussing a couple of results in the area of biomimetic dendrimers, this review highlighted various work on dendrimers whose monomer units are derived from carbohydrates, amino acids, or nucleotides and also several promising biomedical applications under active investigation.

27.6.1 Enhanced Biocompatibility for Drug Delivery

PAMAM dendrimers having poly(ethylene glycol) (PEG) grafts were designed as a novel drug carrier that possesses an interior for the encapsulation of drugs and a biocompatible surface. PEG monomethyl ether with the average MW of 550 or 2000 Da was combined to essentially every chain end of the dendrimer of the third or fourth generation via urethane bond (Kojima et al. 2000). The PEG-attached dendrimers encapsulating anti-cancer drugs adriamycin and methotrexate were prepared by extraction with chloroform from mixtures of the PEG-attached dendrimers and varying amounts of the drugs. Their ability to encapsulate these drugs increased with increasing dendrimer generation and chain length of PEG grafts. Among the PEG-attached dendrimers prepared, the highest ability was achieved by the dendrimer of the fourth generation having the PEG grafts with the average MW of 2,000 Da that could retain 6.5 adriamycin molecules or 26 methotrexate molecules/dendrimer molecule. The methotrexate-loaded PEG-attached dendrimers slowly released the drug in an aqueous solution of low ionic strength. However, in isotonic solutions, methotrexate and doxorubicin were readily released from the PEG-attached dendrimers.

Padilla et al. (2002) described the use of high MW polymers ($>$ 20,000 Da) as soluble drug carriers to improve drug targeting and therapeutic efficacy. They evaluated various dendritic architectures composed of a polyester dendritic scaffold based on the monomer unit 2,2-bis(hydroxymethyl) propanoic acid for their suitability as drug carriers both in vitro and in vivo. These systems are both water-soluble and non-toxic. In addition, the potent anti-cancer drug doxorubicin was covalently bound via a hydrazone linkage to a high MW 3-arm poly(ethylene oxide) (PEO)-dendrimer hybrid. Drug release was a function of pH, and the release rate was more rapid at pH $<$ 6. The cytotoxicity of the DOX-polymer conjugate measured on multiple cancer lines in vitro was reduced but not eliminated, indicating that some active doxorubicin was released from the drug polymer conjugate under physiological conditions. Furthermore, biodistribution experiments show little accumulation of the DOX-polymer conjugate in vital organs, and the serum half-life of doxorubicin attached to an appropriate high MW polymer has been significantly increased when compared to the free drug. Therefore, this new macromolecular system exhibits promising characteristics for the development of new polymeric drug carriers.

Kolhe et al. (2003) attached PEG grafts to the terminal amine groups of the dendrimer and enhanced biocompatibility of higher generation PAMAM dendrimer. Similarly, Bhadra et al. (2003) described the use of uncoated and PEGylated newer PAMAM dendrimers for delivery of an anti-cancer drug 5-fluorouracil. The 4.0 G PAMAM dendrimer was PEGylated using *N*-hydroxysuccinimide-activated carboxymethyl MPEG-5000. The PEGylation of the systems was found to have increased their drug-loading capacity, reduced their drug release rate, and hemolytic toxicity.. TEM study revealed quite uniform surface of the systems. The systems were found suitable for prolonged delivery of an anti-cancer drug by in vitro and blood-level studies in albino rats without producing any significant hematological disturbances. PEG modification has been found to be suitable for modification of PAMAM dendrimers for reduction of drug leakage and hemolytic toxicity. This, in turn, could improve drug-loading capacity and stabilize such systems in the body. The study suggests use of such PEGylated dendrimeric systems as nanoparticulate depot type of system for drug administration (Figure 27.3).

The entrapment of 5-fluorouracil in PEG modification dendrimers significantly increased by 12 times because of more sealing of dendrimeric structure by PEG coating at the peripheral portions of

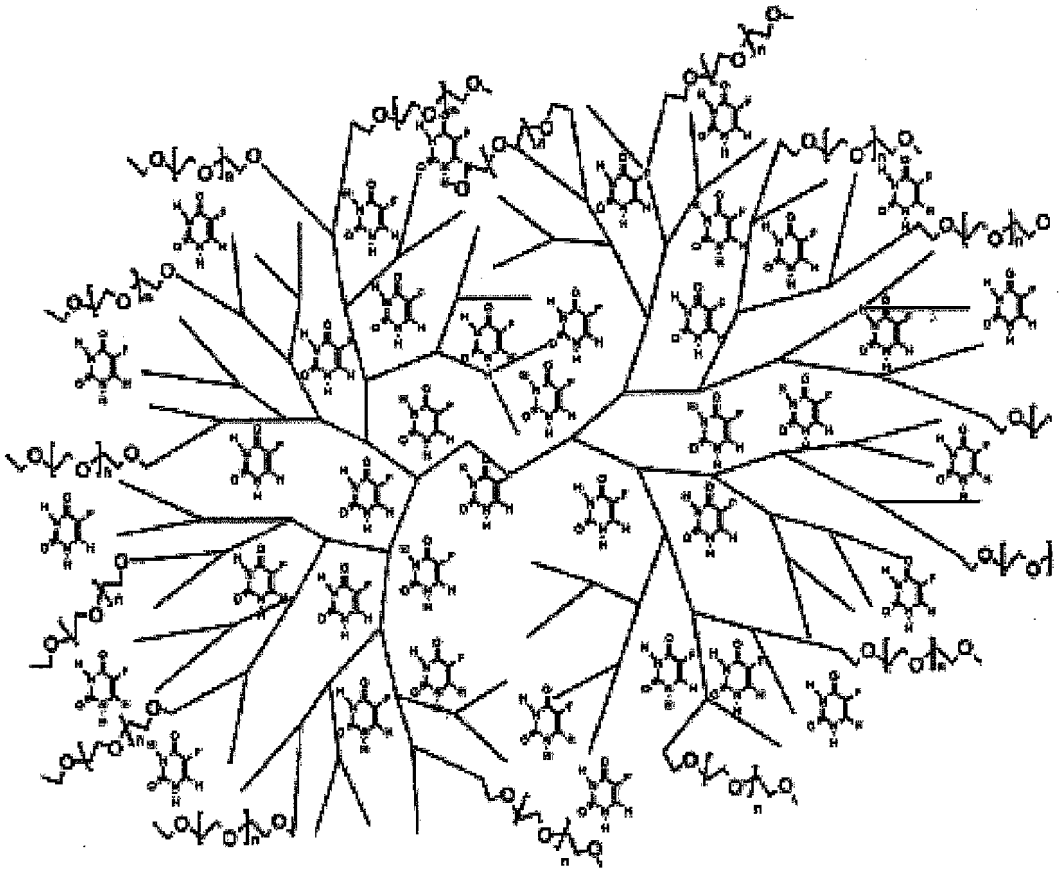

FIGURE 27.3 Chemical structural representation of PEG coated PAMAM loaded with 5-fluorouracil. (Adapted from Bhadra, et al., *Int. J. Pharm.*, 257, 111, 2003. With permission.)

dendrimers as coat that prevented drug release by enhancing complexation probably by steric and electronic effects of the additional functional groups made available by MPEG (Figure 27.4). This, in turn, also reduced the rate of drug release from such systems across dialysis membrane as observed by their release profile. The PAMAM dendrimers on PEGylation behaved similar to

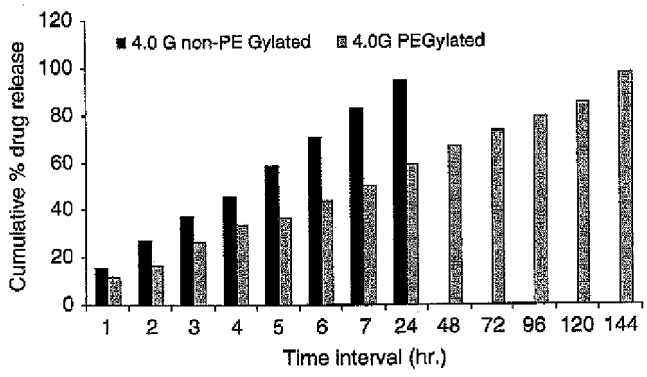

FIGURE 27.4 Comparative cumulative percentage release of 5-fluorouracil from PEG coated and uncoated PAMAM dendrimers. (Adapted from Bhadra, et al., *Int. J. Pharm.*, 257, 111, 2003. With permission.)

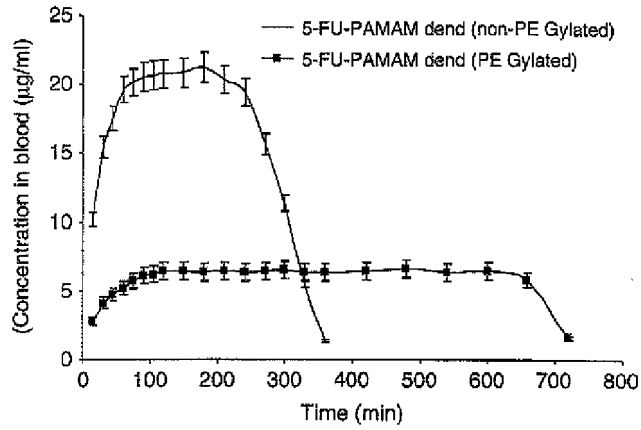

FIGURE 27.5 Blood level data of 5-fluorouracil from PEG coated and uncoated PAMAM dendrimers. (Adapted from Bhadra, et al., *Int. J. Pharm.*, 257, 111, 2003. With permission.)

spherical unimolecular polymer micelles with chains of hydrated methoxy-poly(ethyleneglycol) on its surface as coat. This increased the entrapment capacity of dendrimers and made them act as nanometric containers carrying drugs. Such PEGylated dendrimers can be suggested for sustaining delivery of the drug 5-fluorouracil as also observed by the blood level data where blood level was much prolonged and was detectable up to 12 h. The formulations were found to be following sustained release characteristics for 5-FU (Figure 27.5) as shown by the relative increase in MRT for both non-PEGylated and PEGylated drug-dendrimer complexes as compared to plain drug solution (6.024 and 13.31 times, respectively). The release rate, however, was found to have increased in vivo as compared to in vitro data, possibly because of the metabolism by the enzymes and hydrolysis in the body. The blood level of the drug was found to be lower in case of PEGylated systems than that of non-PEGylated as a result of slower release rates of the drug similar to the trend found in vitro for the drug release. This might also be attributed to better cellular penetration and adhesion properties of the PEG chains of PEGylated systems that might lead to entanglement of such systems within capillary fenestration from which such systems might release drugs acting as nanoparticulate depot type carrier present in blood circulation.

27.6.2 Micellar Means of Enhancing Solubility

Chapman and coworkers prepared amphiphilic copolymers derived from linear PEO and tBoC-terminated poly-$\alpha,\in$-lysine dendrimers. The use of PEO as a platform for the dendrimers synthesis greatly facilitated separation because product up to fourth generation could be precipitated from reaction mixtures by ether. Surface tension method was employed to determine Critical Micelle Concentration (CMC) and micelle formation behavior. The surface of the aggregates was suggested to be highly compact. The existence of transition concentration for dye solubility of dye orange OT by G4 hydramphiphiles in water supports the micellar behavior (Chapman et al. 1994).

Frechet and coworkers also synthesized another novel class of amphiphilic star copolymers. A four-armed PEG star was attached to four polyether dendrons derived from a pentaerythritol core. Results of SEC/VISC and ^{1}HNMR studies indicated the formation of unimolecular micelles in chloroform, tetrahydrofuran, or methanol, but with strikingly different structures. The star copolymers could self organizes into different micellar structures as a function of the environment. A potential application of these stimuli responsive copolymers involves their uses as solvent specific encapsulation agents (Gitsov and Frechet 1996).

Water-soluble dendritic unimolecular micelles as potential drug delivery agents had been explored by Liu et al. (2000) with the hydrophobic core surrounded by hydrophilic shell. These were prepared by coupling dendritic hypercores with PEG-mesylates. The monomeric core that was selected to build it was 4,4-bis(4′hydroxy phenyl) pentanol as this larger monomeric unit provides flexibility to the dendritic structures while contributing to the container capacity of the overall structure. Four generations of dendritic hypercores with 6, 12, 24, and 48 phenolic end groups were prepared. Subsequent coupling reaction with PEG-mesylates afforded four generations of dendritic unimolecular micelles (Gitsov and Fréchet 1993). The container property was demonstrated by pyrene solubilization in aqueous solution. In general, copolymers containing low generation dendrons, especially G1, tended to form unimolecular micelle, whereas G2 and G3 copolymers formed multi molecular micelles, presumably driven by hydrophobic effects and π–π interaction between dendritic blocks. Coexistence of two well-separated peaks in the SEC indicated a slow exchange process between these two species of different sizes.

Ooya, Lee, and Park (2003) developed new methods and pharmaceutical compositions to increase the aqueous solubility of paclitaxel (PTX), a poorly water-soluble drug. Graft and star-shaped graft polymers consisting of PEG400 graft chains increased the PTX solubility in water by three orders of magnitude. Polyglycerol dendrimers (dendriPGs) dissolved in water at high concentrations without significantly increasing the viscosity and at 80 wt% were found to increase the solubility of PTX 10,000-fold. The solubilized PTX was released from graft polymers, star-shaped graft polymers, and the dendriPGs into the surrounding aqueous solution. The release rate was a function of the star shape and the dendrimer generation. The availability of the new graft, star, and dendritic polymers having ethylene glycol units should permit development of novel delivery systems for other poorly water-soluble drugs.

27.6.3 Enhancing Pharmacokinetic Properties

New polyester dendrimer-PEO bow-tie hybrids were evaluated for their potential as drug delivery vehicles and to explore the effect of MW and architecture on their pharmacokinetic properties (Elizabeth et al. 2005). In vitro experiments showed that these polymers were non-toxic to MDA-MB-231 cells and that they degraded to lower MWs at the normal physiological pH of 7.4 and at the mildly acidic pH of 5.0 that may be encountered upon uptake of these molecules by endocytosis and subsequent trafficking to endosomes and lysosomes. Biodistribution studies showed that all carriers with MWs of 40,000 and greater had long plasma circulation times, whereas those with lower MWs were cleared more rapidly with significant quantities excreted in the urine. In general, it was found that the more branched [G-3] polymers exhibited increased circulation times and decreased renal clearance relative to the less branched polymers, a property likely attributable to their decreased flexibility and resulting difficulty in passing through glomerular pores. It was also demonstrated that the more branched structures provide increased levels of steric protection for the payload on the drug-carrying dendron at the core of the molecule. High levels of tumor accumulation were found for two [G-3] polymers in mice bearing subcutaneous B16F10 melanoma. Overall, the features of these new branched carriers include degradability, lack of toxicity, long circulation half-lives, and high levels of tumor accumulation make them very promising polymers for therapeutic applications. High MW polymers have shown promise in terms of improving the properties and the efficacy of low MW therapeutics. However, new systems that are highly biocompatible are biodegradable, have well-defined MW, and have multiple functional groups for drug attachment are still needed. The biological evaluation of a library of eight polyester dendrimer-PEO bow-tie hybrids is described here.

The group of evaluated polymers was designed to include a range of MWs (from 20,000 to 160,000) and architectures with the number of PEO arms ranging from two to eight. In vitro experiments revealed that the polymers were non-toxic to cells and were degraded to lower MW species at pH 7.4 and pH 5.0. Biodistribution studies with ^{125}I-radiolabeled polymers showed that

the high MW carriers (>40,000) exhibited long circulation half-lives. Comparison of the renal clearances for the four-arm versus eight-arm polymers indicated that the more branched polymers were excreted more slowly into the urine, a result attributed to their decreased flexibility. Because of their essentially linear architecture that does not provide for good isolation of the iodinated phenolic moieties, the polymers with two arms were rapidly taken up by the liver. The biodistributions of two long-circulating high MW polymers in mice bearing subcutaneous B16F10 tumors were evaluated, and high levels of tumor accumulation were observed. These new carriers are promising for applications in drug delivery and are also useful for improving the understanding of the effect of polymer architecture on pharmacokinetic properties (Elizabeth et al. 2005).

27.6.4 Dendrimer Use as Transfection Reagents

The recent completion of the human genome sequence has provided promising tools for defining cancer-specific targets, including genes and their expression patterns. In addition, the development in proteomics and achievements of high-throughput screenings offer great potential for discovering effective drugs, including DNA drugs, for gene therapy. Therefore, cancer therapy is no longer limited by the identification of genes; it is limited by an inability to deliver them efficiently to cancer cells, and the bottleneck in cancer gene therapy will soon be shifting from gene discovery to gene delivery (Luo 2004). The goal is to provide adequate amounts of drugs that are close to targeted cells because the actual destination for all gene delivery is the cell nucleus. Only drugs that are close to cells can be internalized. Once inside, intracellular barriers are considerably different. For example, diffusion is no longer the bottleneck. Rather, cytosol survival and nuclear targeting are more important (for non-viral gene delivery). An important and difficult task for cancer gene therapy is increasing the expression level of the therapeutic gene (that is usually toxic) in targeted tumor cells, but at the same time, avoiding its expression in normal tissue (Luo and Saltzman 2000).

Methoxypoly(ethylene glycol)-*block*-poly(L-lysine) dendrimer was designed to form a water-soluble complex with plasmid DNA. The copolymer was synthesized by the liquid-phase peptide synthesis method (Choi et al. 1999). Agarose gel electrophoresis and DNase I protection assay proved that this linear polymer/dendrimer block copolymer spontaneously assembled with plasmid DNA, forming a water-soluble complex that increased the stability of the complexed DNA. Atomic force microscopy of the complex was evaluated at various charge ratios, showing that the copolymer/DNA complex was like a globular shape.

Choi et al. (2000) synthesized and used a barbell-like triblock copolymer, poly(L-lysine) dendrimer-*block*-poly(ethylene glycol)-*block*-poly(L-lysine) dendrimer, for delivery of plasmid DNA by its self-assembly with the structures. A barbell-like ABA-type triblock copolymer, poly(L-lysine) dendrimer-*block*-poly(ethylene glycol)-*block*-poly(L-lysine) dendrimer (PLLD–PEG–PLLD), was synthesized by the liquid-phase peptide synthesis method. The self-assembling complex formation of the third and fourth generation of the copolymer with plasmid DNA was studied. ^{1}H NMR and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) were used for the characterization of the synthesized copolymer. The self-assembling behavior of the third and fourth generations of the copolymer with plasmid DNA was investigated by electrophoretic mobility shift assay, DNase I protection assay, and ethidium bromide exclusion assay. They observed great differences in the self-assembling ability of the third and fourth generations of the polymer. This suggests that the number of positively charged amines per polymer molecule should be an important factor for the potential for self-assembling complex formation with DNA. Atomic force microscopy (AFM) and ζ potentials were used for evaluating the shape, size distribution, and surface charge of the complexes at various charge ratios. From AFM images, it was observed that the shape of the complex was nearly spherical, and its size was about 50–150 nm in diameter. The in vitro cytotoxicity of the copolymer was compared with that of poly(L-lysine), poly(D-lysine), and polyethylenimine.

Luo et al. (2002) demonstrated a simple and successful synthetic approach to devise a highly efficient DNA delivery system with low cytotoxicity and low cost. PAMAM dendrimer is a highly efficient DNA delivery agent when compared to other chemical transfection reagents. Partially degraded, high-generation dendrimers offer even higher efficiency, presumably because of enhanced flexibility of the otherwise rigid dendrimer chains. It was hypothesized that chemical modification of low generation dendrimer with biocompatible PEG chains would create a conjugate of PAMAM core with flexible PEG chains that mimics the fractured high-generation dendrimer and produces high transfection efficiency. Generation 5 PAMAM dendrimer was modified with 3400 MW PEG. The novel conjugate produced a 20-fold increase in transfection efficiency compared with partially degraded dendrimer controls. The cytotoxicity of PEGylated dendrimers was very low. This extremely efficient, highly biocompatible, low-cost DNA delivery system can be readily used in basic research laboratories and may find future clinical applications.

This system can be readily used in basic research laboratories and may be modified and applied in future clinical usage. Furthermore, similar approaches could be adopted with other DNA delivery agents that may create new opportunities for using DNA as a drug via a synthetic delivery route.

Mannisto et al. (2002) investigated the influence of shape, MW, and PEGylation of linear, grafted, dendritic, and branched poly(L-lysine) on their DNA delivery properties. DNA binding, condensation, complex size and morphology, cell uptake, and transfection efficiency were determined. Most poly(L-lysine) condense DNA linear polymers and are at the same time more efficient than most dendrimers. At low MWs of PLL, DNA binding and condensation were less efficient, particularly with dendrimers. PEGylation did not decrease DNA condensation of PLLs at less than 60% (fraction of MW) of PEG. PEGylation sterically stabilized the complexes but did not protect them from interaction with polyanionic chondroitin sulfate. Cell uptake of poly(L-lysine)/DNA complexes was high and PEGylation increased the transfection efficacy. However, overall transfection level of poly(L-lysine) is low possibly as a result of the inadequate escape of the complexes from endosomes or poor release of DNA from the complexes. Physicochemical and biological structure-property relationships of poly(L-lysine) were demonstrated, but no clear correlations between the tested physicochemical determinants (size of complexes, zeta-potentials, condensation of DNA, and the shape of complexes) and biological activities were seen. Intracellular factors and/or still unknown features of DNA complexation may ultimately determine transfection activity with the carriers.

Kim et al. (2004) synthesized and applied a novel triblock copolymer, PAMAM-*block*-PEG-*block*-PAMAM, as a gene carrier. PAMAM dendrimer is proven to be an efficient gene carrier itself, but it is associated with certain problems such as low water solubility and considerable cytotoxicity. Therefore, they introduced PEG to engineer a non-toxic and highly transfection-efficient polymeric gene carrier because PEG is known to convey water-solubility and biocompatibility to the conjugated copolymer. This copolymer could achieve self-assembly with plasmid DNA, forming compact nanosized particles with a narrow size distribution. Fulfilling expectations, the copolymer was found to form highly water-soluble polyplexes with plasmid DNA, showed little cytotoxicity despite its poor degradability, and finally achieved high transfection efficiency comparable to PEI. Consequently, they showed that an approach involving the introduction of PEG to create a tree-like cationic copolymer possesses a great potential for use in gene delivery systems. Logically, this approach could be extended to cancer treatment.

27.6.5 Stimuli Responsive Carriers

Gillies and Frechet (2002) designed and prepared a new polyester dendrimer, PEO hybrid systems, for drug delivery and related therapeutic applications. These systems consist of two covalently attached polyester dendrons where one dendron provides multiple functional handles for the attachment of therapeutically active moieties, whereas the other is used for attachment of solubilizing PEO chains. By varying the generation of the dendrons and the mass of the PEO chains, the MW,

architecture, and drug loading can be readily controlled. The bow-tie shaped dendritic scaffold was synthesized using both convergent and divergent methods with orthogonal protecting groups on the periphery of the two dendrons. PEO was then attached to the periphery of one dendron using an efficient coupling procedure. A small library of eight carriers with MWs ranging from about 20 to 160 kDa were prepared and characterized by various techniques, confirming their well-defined structures.

Gillies, Jonsson, and Frechet (2004) used a pH-responsive micelle system, linear-dendritic block copolymers comprising PEO and either a polylysine or polyester dendron. These were prepared, and hydrophobic groups were attached to the dendrimer periphery by highly acid-sensitive cyclic acetals. These copolymers were designed to form stable micelles in aqueous solution at neutral pH and disintegrate into unimers at mildly acidic pH following loss of the hydrophobic groups upon acetal hydrolysis. Micelle formation was demonstrated by encapsulation of the fluorescent probe Nile Red, and the micelle sizes were determined by dynamic light scattering. The structure of the dendrimer block, its generation, and the synthetic method for linking the acetal groups to its periphery all had an influence on the CMC and the micelle size. The rate of hydrolysis of the acetals at the micelle core was measured for each system at pH 7.4 and pH 5, and it was found that all systems were stable at neutral pH but that they underwent significant hydrolysis at pH 5 over several hours. The rate of hydrolysis at pH 5 was dependent on the structure of the copolymer, most notably the hydrophobicity of the core-forming block. To demonstrate the potential of these systems for controlled release, the release of Nile Red as a model payload was examined. At pH 7.4, the fluorescence of micelle-encapsulated Nile Red was relatively constant, indicating it was retained in the micelle, and at pH 5, the fluorescence decreased, consistent with its release into the aqueous environment. The rate of release was strongly correlated with the rate of acetal hydrolysis and was controlled by the chemical structure of the copolymer. The mechanism of Nile Red release was investigated by monitoring the change in size of the micelles over time at acidic pH. Dynamic light scattering measurement showed a size decrease over time, eventually reaching the size of a unimer, providing evidence for the proposed micelle disintegration.

27.6.6 Alteration of Toxicity

In one of the studies Bhadra et al. (2003) found that PEGylation significantly decreased the hemolysis of red blood cells (RBCs) to below 5% in case of whole generation amine terminated charged dendrimers having about 15.3–17.3% hemolytic toxicity, similar to negligible in case of half generations of carboxylic acid terminated dendrimers. This was due to inhibition of interaction of RBCs with the charged quaternary ammonium ion as determined by interaction with RBCs. The hemolytic toxicity of the dendrimers was enough to preclude its use as a drug delivery system. The toxicity was due to the poly-cationic nature of the PAMAM dendrimers that was also responsible for their cytotoxicity (Malik et al. 2000), particularly in the case of whole generation amine terminated charged dendrimers, but not in the case of half generations of carboxylic acid terminated dendrimers.

The PEGylation of the dendrimers were found to have also considerably lower rate of hemolysis of the RBCs because of significantly reduced interaction of RBCs with the charged quaternary ammonium ion that is generally present on the amine terminated whole generations of dendrimers. Also, the hematological study was undertaken to assess the relative effects of the PEGylated and non-PEGylated systems as compared to the plain drug on various blood parameters. The blood parameters undergoing major changes as to normal values of blood levels are RBC count, WBC count, and differential lymphocytes count. The RBC count of non-PEGylated 5-FU-dendrimers was found to have decreased below normal values by about $1\times10^6/\mu l$ RBCs to $2\times10^6/\mu l$ RBCs as against similar PEGylated systems. The WBC count of non-PEGylated 5-FU-dendrimer complex increased by $3\times10^3/\mu l$ cells to $4\times10^3/\mu l$ cells as compared to normal values. However, for 5 FU-PEGylated dendrimer complexes, the increase was by $1\times10^3/\mu l$ cells to $1.5\times10^3/\mu l$ WBCs

as compared to normal count in controlled group. The increase in WBC count is significant in case of non-PEGylated systems. Similarly, relatively greater increase in lymphocyte count was observed by non-PEGylated dendrimer-drug complexes that were by about $2\times10^3/\mu l$ cells. This was similar to blood toxicity and cytotoxicity effects of acrylates nanoparticulates (Malik et al. 2000), which are known to be stimulating the macrophage level and WBC count. This was found to be true in the case of these non-PEGylated drug-dendrimeric systems but not in case of PEGylated systems, conforming the trends of the PEGylated carriers (Veronese et al. 1989; Papahadjopoulus et al. 1991; Phillips et al. 1999) undergoing lesser phagocytic uptake.

Neerman et al. (2004) also showed that dendrimers based on melamine could reduce the organ toxicity of solubilized cancer drugs administered by intraperitoneal injection. Methotrexate and 6-mercaptopurine, both FDA approved anti-cancer drugs, are known hepatotoxins. The solubility of these molecules can be increased by mixing them with a dendrimer based on melamine. C3H mice were administered subchronic doses of methotrexate or 6-mercaptopurine with and without a solubilizing dendrimer. Forty-eight hours after dosing, the mice were sacrificed, and serum was collected for biochemical analyses. The levels of alanine transaminase, ALT, were used to probe liver damage. When the drugs are encapsulated by the dendrimer, a significant reduction in hepatotoxicity is observed; ALT levels from the rescued groups (drug + dendrimer) were 27% (methotrexate) and 36% (6-mercaptopurine) lower than those of animals treated with the drug alone.

Initial studies of a generation 3, cationic dendrimer bearing 24 primary amino groups revealed that this dendrimer was toxic both in vitro and in vivo. Extensive liver damage was observed when mice were challenged with subchronic doses of this dendrimer. Other groups hypothesized that surface group charge was the determining factor in predicting a dendrimer's toxicity as cationic surface groups will electrostatically adhere to cell surface, inducing bulk adhesion that leads to the cell's demise. Surface group modification of a generation 3, cationic dendrimer bearing 48 primary amino groups to possess anionic or neutral surface groups had a significant impact on both in vitro and in vivo toxicity. The introduction of neutral PEG groups afforded the most protection in vitro and in vivo. Shielding of the dendrimer's charged groups attenuates the bulk adhesion of these molecules, leading to a decrease in the toxicity of these dendrimers. It appeared that PEGylation afforded the most protection against toxicity of dendrimers based on melamine. As a result, the candidate vehicle was subjected to a series of analyses. Albumin interaction studies showed that this dendrimer interacted with albumin that could have a profound effect on the dendrimer's pharmacokinetic parameters. In vitro drug release profiles of the anti-cancer drugs methotrexate and paclitaxel revealed that methotrexate was rapidly released while paclitaxel showed a more controlled, sustained release. This discrepancy in drug release was attributed to disparities in the drug's hydrophobicity; the more hydrophobic drug, paclitaxel, formed a stronger association with the dendrimer's hydrophobic core. As a result, release was more controlled. In terms of the in vitro cytotoxicity of these dendrimer/drug formulations, free drug was more toxic than the dendrimer-associated drug. This was attributed to differences in the cellular uptake of free versus dendrimer-associated drug. To assess if a difference in cellular uptake existed between free and dendrimer associated substrates, cells exposed to free rhodamine B showed higher intracellular levels of dye than those cells exposed to dendrimer-associated rhodamine B. The preliminary in vivo biodistribution screen of the Cy5.5-labeled dendrimer showed that the dendrimer was still present in the body 24 h post injection while exhibiting a high degree of liver accumulation after such time (https://txspace.tamu.edu/dev-xml/bitstream/1969.1/5330/1/etd-tamu-2005A-TOXI-Neerman.pdf).

Chen et al. (2004) designed a small library of dendrimers prepared from a common precursor that is available in 5 g scale in five linear steps at 56% overall yield. The precursor is a generation three dendrimer that displays 48 peripheral sites by incorporating AB4 surface groups. Manipulation of these sites provided six dendrimers that vary in the chemistry of the surface group (amine, guanidine, carboxylate, sulfonate, phosphonate, and PEGylated) that were evaluated for hemolytic potential and cytotoxicity. Cationic dendrimers were found to be more cytotoxic and hemolytic

than anionic or PEGylated dendrimers. The PEGylated dendrimer was evaluated for acute toxicity in vivo. No toxicity, neither mortality nor abnormal blood chemistry, based on blood urea nitrogen levels or alanine transaminase activity was observed in doses up to 2.56 g/kg i.p. and 1.28 g/kg intavenously.

27.6.7 Effective Photodynamic Therapy

Photosensitizers play a crucial role in the photodynamic therapy (PDT) of cancer. Zhang et al. (2003) observed that the use of PIC micelles as a delivery system reduced the dark toxicity of the cationic dendrimer porphyrin, probably because of the biocompatible PEG shell of the micelles. They compared relatively low cellular uptake of dendrimer porphyrin $[NH_2CH_2CH_2NHCO]_{32}$-DPZ_n incorporated in the PIC micelle as was observed, yet the latter exhibited enhanced photodynamic efficacy on the Lewis Lung Carcinoma (LLC) cell line. In this study, a third-generation aryl ether dendrimer porphyrin with 32 primary amine groups on the periphery, $[NH_2CH_2CH_2NHCO]_{32}DPZ_n$, and pH-sensitive, polyion complex micelles (PIC) composed of the porphyrin dendrimer and PEG-b-poly(aspartic acid) were evaluated as new photosensitizers (PSs) for PDT in the LLC cell line. The preliminary photophysical characteristics of $[NH_2CH_2$-$CH_2NHCO]_{32}DPZ_n$ and the corresponding micelles were investigated. Electrostatic assembly resulted in a red-shift of the Soret peak of the porphyrin core and the enhanced fluorescence.

Jang et al. (2005) described a supramolecular nanocarrier of anionic dendrimer porphyrins with cationic block copolymers modified with PEG to enhance intracellular photodynamic efficacy.

27.6.8 Dendritic Gels

Luman, Smeds, and Grinstaff (2003) developed a process of high-yield convergent synthesis of dendrons, dendrimers, and dendritic-linear hybrid macromolecules composed of succinic acid, glycerol, and PEG. This convergent synthesis relies on two orthogonal protecting groups, namely, the benzylidene acetal (bzld) for the protection of the 1,3-hydroxyls of glycerol and the tert-butyldiphenylsialyl (TBDPS) ester for protection of the carboxylic acid of succinic acid. These novel polyester dendritic macromolecules are entirely composed of building blocks known to be biocompatible or degradable in vivo to give natural metabolites. Derivatization of the dendritic periphery with a methacrylate affords a polymer that can be subsequently photo-cross-linked. The three-dimensional cross-linked gels formed by ultraviolet irradiation are optically transparent with mechanical properties dependent on the initial cross-linkable dendritic macromolecule.

27.6.9 Ligand-Based Drug Delivery

Architectural features of synthetic ligands were systematically varied to optimize inhibition of mast cell degranulation initiated by multivalent crossing of IgE-receptor complexes Baird et al. (2003). A series of ligands were generated by end-capping PEG polymers and amine-based dendrimers with the hapten 2,4-dinitrophenyl (DNP). These were used to explore the influence of polymeric backbone length, valency, and hapten presentation on binding to anti-DNP IgE and inhibition of stimulated activation of RBL cells. Monovalent MPEG(5000)-DNP (IC(50) = 50 nM), bivalent DNP-PEG(3350)-DNP (IC(50) = 8 nM), bismonovalent MPEG(5000)-DNP(2) (IC(50) = 20 nM), bisbivalent DNP(2)-PEG(3350)-DNP(2) (IC(50) = 3 nM), and DNP(4)-dendrimer ligands (IC(50) = 50 nM) all effectively inhibit cellular activation caused by multivalent antigen, DNP-bovine serum albumin. For different DNP ligands, evidence is available for more effective inhibition because of preferential formation of intra-IgE cross-links by bivalent ligands of sufficient length, self-association of monovalent ligands with longer tails, and higher probability of binding for bisvalent ligands. They also showed that larger DNP(16)-dendrimers of higher valency trigger degranulation by cross-linking

IgE-receptor complexes, whereas smaller DNP-dendrimers are inhibitory. Therefore, features of synthetic ligands can be manipulated to control receptor occupation, aggregation, and inhibition of the cellular response.

27.6.10 Increasingly Effective Boron Neutron Capture Therapy

A recent methodology in cancer treatment is the boron neutron-capture therapy (BNCT) (Hawthorne 1993). In this therapy, the generation of cytotoxic and energetic products from nuclear fission reactions of low-energy neutrons and ^{10}B nuclei is used to destroy malignant cells. An efficient agent for the therapy is water-soluble and has a high local density of boron clusters, requirements that are met for several synthesized boron-containing and water-soluble dendrimers. Qualmann et al. (1996a, 1996b) have, in addition, introduced antigen selectivity by coupling a lysine-based boronated dendrimer to antibody fragments (Fabϕ). In these agents, the attachment of a poly(ethylene glycol)(PEG) moiety is necessary to keep the conjugates water-soluble. The covalent nature of the boronated Fabϕ fragments leads to a better stability of these conjugates as compared to, for example, borate-coated polystyrene beads.

Successful treatment of cancer by boron neutron capture therapy (BNCT) requires the selective delivery of ^{10}B to constituent cells within a tumor. The expression of the folate receptor is amplified in a variety of human tumors and potentially might serve as a molecular target for BNCT. Shukla et al. (2003) investigated the possibility of targeting the folate receptor on cancer cells using folic acid conjugates of boronated poly(ethylene glycol) (PEG) containing third generation PAMAM dendrimers to obtain ^{10}B concentrations necessary for BNCT by reducing the uptake of these conjugates by the reticuloendothelial system. First, they covalently attached 12–15 decaborate clusters to third generation PAMAM dendrimers. Varying quantities of PEG units with varying chain lengths were then linked to these boronated dendrimers to reduce hepatic uptake. Among all prepared combinations, boronated dendrimers with 1–1.5 PEG(2000) units exhibited the lowest hepatic uptake in C57BL/6 mice (7.2–7.7% injected dose (ID)/g liver). Therefore, two folate receptor-targeted boronated third generation PAMAM dendrimers were prepared: one containing approximately 15 decaborate clusters and approximately 1 PEG(2000) unit with folic acid attached to the distal end, and the other containing approximately 13 decaborate clusters with, approximately one PEG(800) unit with folic acid attached to the distal end. In vitro studies using folate receptor (+) KB cells demonstrated receptor-dependent uptake of the latter conjugate. Biodistribution studies with this conjugate in C57BL/6 mice bearing folate receptor (+) murine 24JK-FBP sarcomas resulted in selective tumor uptake (6.0% ID/g tumor), but also high hepatic (38.8% ID/g) and renal (62.8% ID/g) uptake, indicating that attachment of a second PEG unit and/or folic acid may adversely affect the pharmacodynamics of this conjugate.

27.6.11 Improved MRI Contrast Agents

Macromolecules conjugated with polyethylene glycol (PEG) acquire more hydrophilicity, resulting in a longer half-life in circulation and lower immunogenicity. Kobayashi et al. (2001) synthesized two novel conjugates for MRI contrast agents from a generation-4 polyamidoamine dendrimer (G4D), 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M), and one or two PEG molecules with a MW of 20,000 Da (PEG(2)-G4D-(1B4M-Gd)(62) (MW: 96 kD), PEG(1)-G4D-(1B4M-Gd)(63) (MW: 77 kD). Their pharmacokinetics, excretion, and properties as vascular MRI contrast agents were evaluated and compared with those of G4D-(1B4M-Gd)(64) (MW: 57 kD). PEG(2)-G4D-(1B4M-Gd)(62) remained in the blood significantly longer and accumulated significantly less in the liver and kidney than the other two preparations ($P<0.01$). Although the blood clearance was slower, PEG(2)-G4D-(1B4M-Gd)(62) was excreted more readily without renal retention than the other two preparations. The positive effects of PEG

conjugation on a macromolecular MRI contrast agent were found to be prolonging retention in the circulation, increased excretion, and decreased accumulation in the organs.

27.7 CONCLUSIONS

Nanotechnology refers to the interactions of cellular and molecular components and engineered materials—typically clusters of atoms, molecules, and molecular fragments—at the most elemental level of biology. Such nanoscale objects—typically, though not exclusively, with dimensions smaller than 100 nm—can be useful by themselves or as part of larger devices, containing multiple nanoscale objects. At the nanoscale, the physical, chemical, and biological properties of materials fundamentally differ, and often nanotechnology can change the very foundations of cancer diagnosis, treatment, and prevention. The novel nanodevices are capable of many clinically important functions, including detecting cancer at its earliest stages, pinpointing its location within the body, delivering anti-cancer drugs specifically to malignant cells, and in killing malignant cells. As these nanodevices are evaluated in clinical trials, researchers envision that nanotechnology will serve as multifunctional tools that will not only be used with any number of diagnostic and therapeutic agents, but will also change the very foundations of cancer diagnosis, treatment, and prevention (NCI 2004).

Such nanoparticulate carriers also include PEG conjugated dendrimers. These are globular, hyperbranched polymers possessing a high concentration of surface functional groups and internal cavities. These unique features make them very useful in many biomedical applications, especially as carrier molecules. There are many such technological opportunities associated with the dendrimers. Starpharma-like companies used these dendrimers to build large active compounds that present a polyvalent array to receptors, and others platform opportunities for the delivery of small molecules as a toolbox for rational drug design and other nanotechnology applications in life sciences. There are many product opportunities in dendrimers and their use such as with VivaGel™, a topical microbicide gel for prevention of HIV and other STDs in women. They can act as novel chemotherapeutic agents, angiogenesis inhibitors, and they can be used for targeting a range of tropical and exotic diseases and as bio-defense agents in addition to their use for targeting a broad range of viral, respiratory, and cancer-like diseases in the future. Development of drug delivery systems with low toxicity and low cost is the key to a nanaotechnological approach to cancer treatment, including DNA-based concepts. Targeting the drugs to specific receptors exclusively expressed by tumor cells also appears to be an approach worth exploring. This biochemical approach is expected to pay rich dividends in the chemotherapy of cancer. Cytosol survival of anti-cancer agents and folate receptor-based approaches also hold promise. Carbon nanotubes offer yet another attractive possibility.

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